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Efficient Selection of Hybrids by Protoplast Fusion Using Drug Resistance Markers and Reporter Genes in *Saccharomyces cerevisiae*

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We have developed a selection system for hybrids by protoplast fusion using dominant selective drug resistance markers, Tn*601*(*903*) against geneticin and *AUR1-C* against aureobasidin A, and reporter genes, *ADH1*p-*PHO5*-*ADH1*t and *CLN2*p-*CYC1*-*lacZ*, in *Saccharomyces cerevisiae*. To examine the effectiveness of this system, plasmids with each marker and reporter gene were introduced into auxotrophic sake yeasts. From the resulting transformants, eight colonies were screened by protoplast fusion in combination with the drug resistance markers and the reporter genes. Among them, seven strains were judged as hybrids between parental strains by analysis of growth on a minimal medium. This selection system was applied to wine yeasts having no genetic markers. Six strains were regarded as hybrids between parental strains by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) analysis of the *MET2* gene and by karyotype analysis using a contour-clamped homogeneous electric field (CHEF). We propose that the plotoplast fusion using dominant selective geneticin- and aureobasidin A-resistance markers and reporter genes is useful for the selection of hybrids from wine yeasts, which are homothallic and have low sporulation ability.

[**Key words:** protoplast fusion, drug resistance markers, reporter genes]

The improvement of industrial strains (*Saccharomyces cerevisiae*) by crossing has been difficult because most of them neither mate nor sporulate. We have developed a method for the direct selection of mating-competent clones from sake yeasts (1) and a mass mating method in combination with resistance markers for the selection of hybrids from homothallic yeasts (2). However, these methods are virtually impracticable for most wine and bottom fermentation beer yeasts that are homothallic and have low sporulation ability. For these strains, breeding via hybridization may be practicable by protoplast fusion. The fusion products were selected mainly by the complementation of auxotrophic strains. This approach is not applied to industrial strains because they are prototrophic. Protoplast fusion has been used in combination with respiratory deficiency and nutritional requirement as selective markers to breed killer wine yeasts (3) or beer yeasts that have high productivity of esters, high tolerance to ethanol and high osmotolerance (4). Homothallic wine yeasts were also constructed by this method using inherent dominant selective drug resistance markers such as chloramphenicol (5) or geneticin (G418) (6) resistance. A biochemical inhibitor, *N*-ethylmaleimide, was used to inactivate protoplasts from yeast cells without genetic markers in protoplast fusion and inactivated protoplasts were fused with untreated protoplasts having genetic markers to obtain hybrids (7). However, if the strains to be hybridized have no nutritional requirement and inherent selective markers, such selective markers must be conferred on them prior to the hybridization process because it is almost impossible to select hybrids without using such markers.

Dominant selective markers are obviously useful for selecting hybrids when the protoplast fusion method is employed because the Tn*601*(*903*) and *AUR1-C* genes of *S. cerevisiae* are useful dominant selective markers for selecting hybrids by a mass mating method when they are ligated into a single copy vector in combination with geneticin and aureobasidin A (2). In this study, for the selection of hybrids by protoplast fusion, plasmids that contained the drug resistance gene, Tn*601*(*903*) or *AUR1-C*, and the reporter gene, *CLN2*p-*CYC1*-*lacZ* or *ADH1*p-*PHO5*-*ADH1*t, were constructed. Hybrids between transformants with each plasmid acquire resistance to both drugs, and their colonies exhibit a blue color on a plate for the detection of β-galactosidase activity and a red color on a plate when stained for acid phosphatase activity. We demonstrate the effectiveness of protoplast fusion in combination with drug resistance markers and reporter genes for selecting hybrids from wine yeasts as this method does not require auxotrophic or respiratory-defective strains.

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MATERIALS AND METHODS

Yeast strains and plasmids The *S. cerevisiae* sake yeasts, K7-U and K9-T (8), and wine yeasts, EC-1118 and UCD530 (9), were used in this study. K7-U and K9-T are uracil (*ura3/ura3*) and tryptophan (*trp1/trp1*) auxotrophic mutants, respectively. Plasmid pIB302 was constructed by blunt-ending the *Bam*HI and *Hap*I ends in pSH39 (10) and connecting with *Xho*I and *Xba*I linkers, respectively. Plasmid pIB304 (Fig. 1) was constructed by inserting a 1.5-kbp *Xho*I-*Xba*I fragment containing the *PHO5* gene from pIB302 into the *Xho*I-*Xba*I gap of pAUR123 (GenBank accession no. AB012284; Takara Shuzo, Kyoto). Plasmid 340 (Fig. 1) was constructed by inserting a 2.5-kbp *Sal*I-*Xba*I fragment having a G418 resistance gene from plasmid pKO6 (2) into the *Sal*I-*Xba*I gap of plasmid pΔ5'728 (11).

Protoplast formation and fusion The method described by Harashima *et al.* (12) was used with slight modification. Cells of the fusion partners with plasmids were grown in 5 ml of YPAD medium (13) with 0.2 mg/ml G418 or 0.2 μg/ml AbA at 30°C overnight. Culture solutions (200 or 400 μl) were inoculated into 10 ml of YPAD medium and cultivated for 4.5 to 5 h at 30°C. Cells were collected by centrifugation and pretreated with 1 ml of 0.1 M sodium citrate buffer (pH 5.8) containing 1.2 M sorbitol, 0.01 M EDTA (SSE buffer), and 0.5% 2-mercaptoethanol for 30 min at 30°C. Cells were washed with SSE buffer and suspended in 4 ml of the same SSE buffer containing 0.4 mg of Zymolyase 100T (Seikagaku, Tokyo). The cell suspension was incubated for 1 h at 30°C. Protoplasts were washed with two 2-ml portions of 1.2 M sorbitol solution containing 10 mM $CaCl_2$ and resuspended by the addition of 0.2 ml of the same solution to the cell pellet after centrifugation at 550×*g* for 5 min. Each 0.1 ml of the protoplast suspension from two different strains was mixed and then 2 ml of 20% polyethylene glycol 4000 (Wako Pure Chemical Industries, Osaka) solution in 10 mM Tris hydrochloride buffer (pH 7.5) containing 10 mM $CaCl_2$ was added. The reaction mixture was incubated at 25°C for 30 min and protoplasts were harvested, resuspended in 0.2 ml of YPAD medium supplemented with 1.2 M sorbitol, and incubated at 30°C for 1 h. Then, 8 ml of YPM medium (1% yeast extract, 2% peptone, 2% maltose) containing 1.2 M sorbitol and 3% agar (Difco Laboratories, Detroit, MI, USA) at 46°C was poured into the protoplast suspension and mixed gently. The above mixture was poured onto the surface of an agar plate of YPDM medium (1% yeast extract, 2% peptone, 1% dextrose, 1% maltose, 2% agar) containing 1.2 M sorbitol, 0.5 mg/ml G418 and 0.3 μg/ml AbA for sake yeasts or 0.3 mg/ml G418 and 0.7 μg/ml AbA for wine yeasts. The plate was incubated at 30°C for 3 to 6 d. Colonies were grown on YPDM medium used for the selection of hybrids without sorbitol and then transferred to SD medium (13) for sake yeasts.

Genetic and biochemical methods *S. cerevisiae* and *Escherichia coli* cells were transformed as described by Ito *et al.* (14) and Sambrook and Russell (15). The acidic phosphatase (APase; EC 3.1.3.2) activity of *S. cerevisiae* colonies was detected by staining based on a diazo-coupling reaction (16) on a YPD plate (13) containing 0.3 μg/ml AbA for sake yeasts, in which the *PHO3* and *PHO5* genes are defective. For wine yeasts, a YPD plate that contained AbA and 2 mM thiamin was used because thiamin represses the expression of the *PHO3* gene (17, 18). The β-galactosidase (EC 3.2.1.23) activity of *S. cerevisiae* colonies was detected by a 5-bromo-4-chloro-3-indolyl-D-galactoside (X-Gal) indicator plate (13) containing 0.5 mg/ml G418.

Separation of chromosomal DNA by pulse-field gel electrophoresis The methods used for the preparation and manipulation of chromosomal DNAs were described by Sheehan and Weiss (19). Chromosomal DNA was separated by a contour-clamped homogeneous electric field (CHEF) DR-II (Bio-Rad, Hercules, CA, USA) in 0.5×Tris–borate–EDTA (TBE) buffer, 6 V/cm at 14°C, the pulse time being changed linearly from 24 to 73 s within 28 h.

Polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) analysis The PCR-RFLP method of the *MET2* region was described by Mansneuf *et al.* (20). Amplification reactions were performed with a PTC-200 Peliter Thermal Cycler (MJ Research, Watertown, MA, USA) under the following conditions: a 50 μl reaction mixture was prepared with 2.5 units of Takara Ex (Takara) Taq DNA polymerase, 5 μl of 10×Ex Taq buffer (Mg^{2+}, 20 mM), 50 pmol of each primer, 2.5 mM of each dNTP and 0.5 μg of a template DNA obtained by a 10-min DNA preparation (13). The sequences of primers OLI147 and OLI148 are 5'-AATCGAAAACGCTCCAAGAG-3' and 5'-TGCACCAGG CAGAATGTCTT-3', respectively. The PCR conditions were modified as follows: a preliminary step of 1 min at 94°C; 35 cycles of 20 s at 94°C, 30 s at 55°C, and 1 min (increasing 1 s per cycle) at

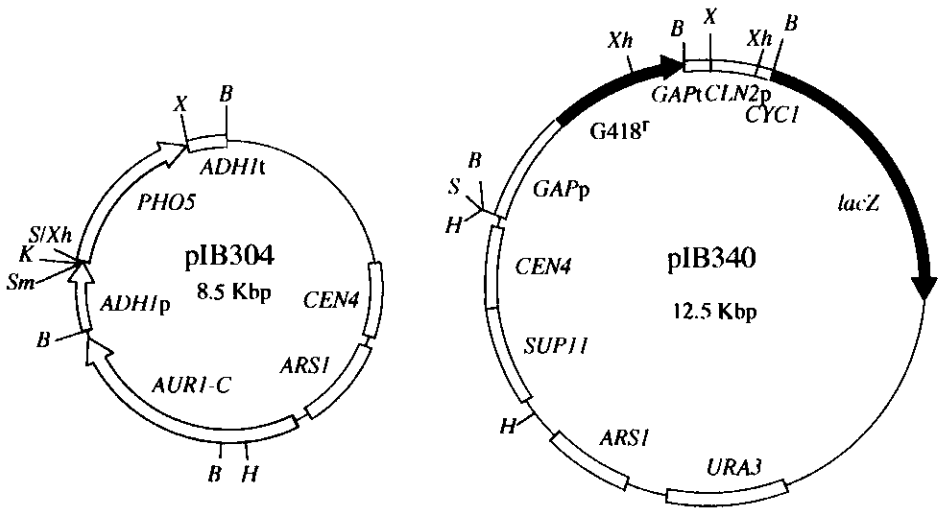

FIG. 1. Structures of plasmid pIB304 having the *AUR1-C* gene and *ADH1*p-*PHO5*-*ADH1*t reporter gene, and plasmid pIB340 harboring the G418-resistance gene and *CLN2*p-*CYC1*-*lacZ* reporter gene. Open boxes indicate DNA fragments of *S. cerevisiae*. Closed boxes represent DNA fragments of *Escherichia coli*. Thin lines indicate DNA fragments originating from the plasmid pBLUESCRIPT (Stratagene, La Jolla, CA, USA). *ADH1*p and *ADH1*t represent, respectively, the promoter and terminator of the *ADH1* gene of *S. cerevisiae*. *CLN2*p represents the promoter of the *CLN2* gene of *S. cerevisiae*. *GAP*p and *GAP*t represent, respectively, the promoter and terminator of the *GAP* gene of *S. cerevisiae*. G418^r represents the geneticin resistance gene. *B*, *Bam*HI; *H*, *Hin*dIII; *K*, *Kpn*I; *S*, *Sal*I; *Sm*, *Sma*I; *X*, *Xba*I; *Xh*, *Xho*I.

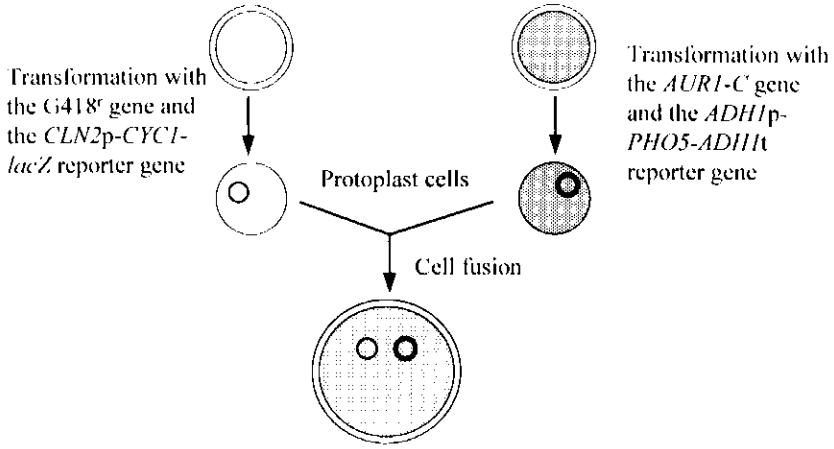

FIG. 2. Selection of hybrids obtained by protoplast fusion using the drug resistance genes and reporter genes. Strains were transformed with the plasmid having G418^r and *CLN2*p-*CYC1-lacZ* or *AUR1-C* and *ADH1*p-*PHO5-ADH1*t. The resultant transformants were hybridized by protoplast fusion. Viable colonies were then tested for their ability to grow on plates containing G418 and aureobasidin A, and the expression of reporter genes. When colonies were resistant to both of these drugs and expressed both reporter genes, they were judged as hybrids between parental strains. Small circles indicate plasmid pIB304 (thick lined circles) or pIB340 (thin lined circles).

72°C; and a final extension step of 10 min at 72°C. Aliquots of PCR products were digested. Amplified fragments and restriction fragments were analyzed by electrophoresis on a 1.8% agarose gel in 0.5×TBE buffer. PCR markers (Novagen, Darmstadt, Germany) were used as DNA molecular markers.

Relative DNA content The relative DNA content was analyzed using a flow cytometer (Becton Dickinson, Sparks, MD, USA). Yeast cells were grown to the logarithmic phase in YPD medium. The cells which were fixed in 70% (v/v) ethanol for 30 min at room temperature were washed with 50 mM Na citrate (pH 7.4) and then treated with 0.25 mg/ml RNase in 50 mM Na citrate (pH 7.4) for 1 h at 50°C and with 100 μg/μl Protease K in 50% glycerol for 1 h at 50°C. They were stained with 8 μg/μl propidium iodide (PI) in 50 mM Na citrate (pH 7.4) overnight at 4°C.

Biochemical methods The methods used for the preparation and manipulation of DNAs were as described by Sambrook and Russell (15). Southern hybridization was performed by the Gene image 3′-oligolabelling and CDP-star detection system (Amersham, Piscataway, NJ, USA).

RESULTS AND DISCUSSION

Fusion of protoplasts with dominant selective markers and reporter genes We devised a method, as illustrated in Fig. 2, for selecting hybrids from yeast cells. It is considered a problem that protoplast cells are more sensitive to drugs than vegetative ones. Two solutions were adopted. First, drugs were added into an agar plate of a regeneration medium because in fungal transformation using protoplast cells, benomyl, which is used for the selection of transformants, is added into a regeneration agar plate (21). Second, the combination of carbon sources was examined. The effect of drugs on *S. cerevisiae* cells is variable depending on the carbon source in the medium (22). When only glucose was used as the carbon source in the medium, the sensitivity of protoplast cells to drugs was very low, and when only maltose was used, the sensitivity was very high (data not shown). From this observation, YPM was used as the medium mixed with protoplast cells and YPDM as the regeneration medium with drugs.

Using the auxotrophic sake yeasts K7-U (*ura3/ura3*) and K9-T (*trp1/trp1*), we tested whether this method is useful for the selection of hybrids obtained by protoplast fusion. K7-U and K9-T were transformed with plasmids pIB340 and pIB304, respectively. Among the resulting transformants, colonies of K7-U with plasmid pIB340 carrying G418^r and the *CLN2*p-*CYC1-lacZ* reporter gene exhibited a blue color on X-Gal indicator plates having G418, and those of K9-T with plasmid pIB304 harboring the *AUR1-C* gene and *ADH1*p-*PHO5-ADH1*t reporter gene exhibited a red color when stained for APase activity on a YPD plate containing AbA. These results indicate that drug resistance genes and reporter genes in both plasmids function normally. Protoplasts from the resulting transformants were fused as described in Materials and Methods. Eight colonies were found on a selection medium.

Evaluation of fusion products To confirm whether the fusion products retain both plasmids, they were grown on a selection medium without sorbitol and examined for the expression of *ADH1*p-*PHO5-ADH1*t and *CLN2*p-*CYC1-lacZ* reporter genes by the plate analyses. All colonies grew on the medium and exhibited a blue color on X-Gal indicator plates containing G418 and a red color when stained for APase activity on a YPD plate having AbA indicating that all of them had both plasmids pIB304 and pIB340. If the colonies are hybrids between K7-U and K9-T, they would grow on SD medium because each auxotrophy was complemented. In order to cure both plasmids from fusion products, they were cultivated in YPAD medium overnight at 30°C and tested for growth on SD medium after observing that the colonies did not grow on a YPAD plate containing G418 or AbA. Seven fusion products were judged to be hybrids between K7-U and K9-T because they grew on SD medium. The remaining one, which did not grow on SD medium, grew on a ura$^-$ plate but not on a trp$^-$ plate. How-

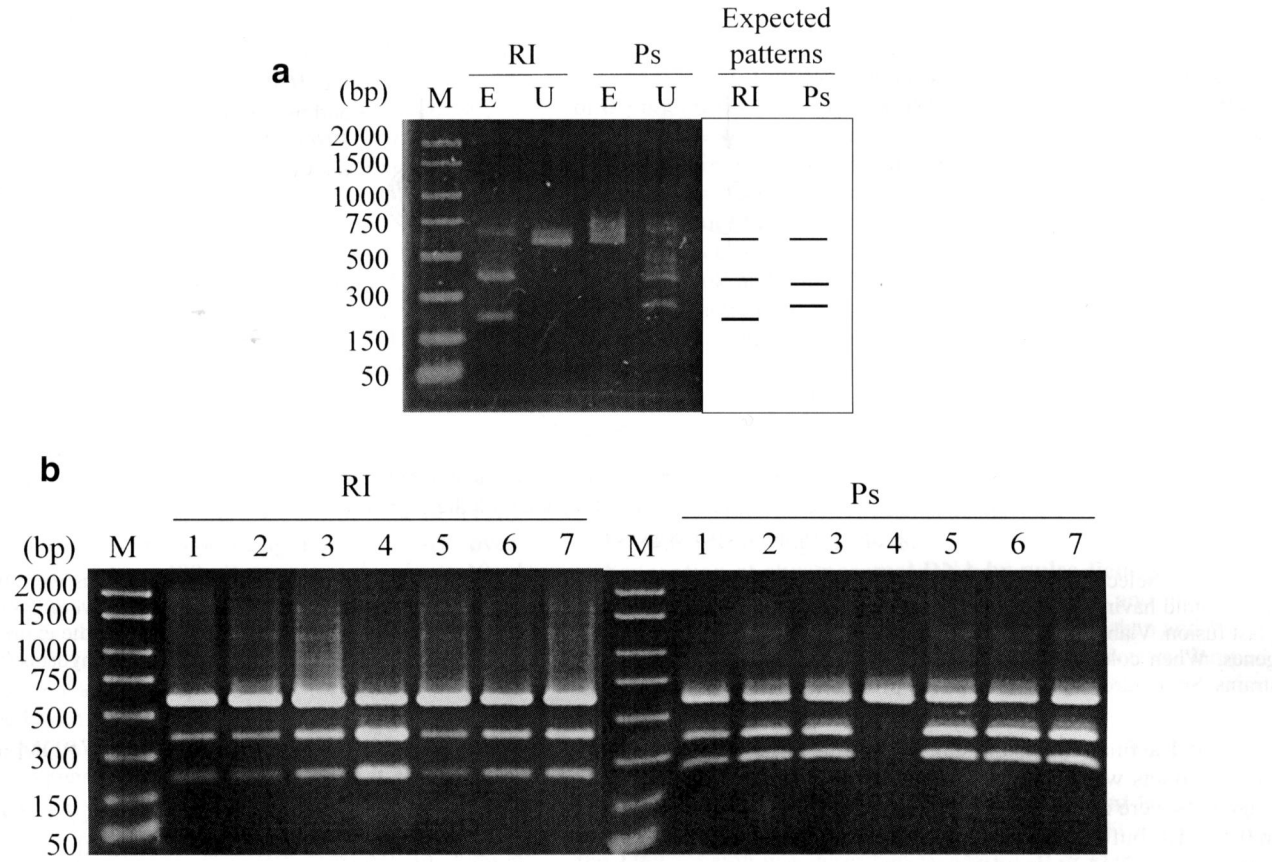

FIG. 3. PCR/RFLP of the *MET2* gene. (a) Analysis of parental strains. Samples from EC-1118 (E) and UCD530 (U) were digested by *Eco*RI (RI) or *Pst*I (Ps). (b) Analysis of fusion products. Samples from fusion products were digested by RI or Ps. The numerals 1 to 7 show strain numbers. Lane M, PCR markers (Novagen, Darmstadt, Germany) were run in parallel as size markers.

ever, it may have a part of the relevant chromosomes from parental strains. Four hybrids were subcultured 20 times to test whether they were stable as fusion products and then they were examined for auxotrophy. They showed the URA^+ and TRP^+ phenotype, indicating that these hybrids are stable.

Selection of hybrids from wine yeasts These observations suggest that drug resistance genes and reporter genes are useful for the selection of hybrids by protoplast fusion of industrial yeasts. Using this method, we tried to obtain hybrids by protoplast fusion in wine yeasts. EC-1118 and UCD530 were transformed with plasmids pIB304 and pIB340, respectively. Protoplasts from resultant transformants were fused. Ten colonies were found on a selection medium. To confirm whether fusion products retain both plasmids, they were grown on a selection medium without sorbitol and examined for the expression of the *ADH1*p-*PHO5-ADH1*t and *CLN2*p-*CYC1-lacZ* reporter genes by plate analyses. Seven colonies grew on the medium and the expression of both reporter genes was observed.

It has been reported that in wine yeasts, PCR/RFLP analysis of the *MET2* gene is a reliable and fast technique for distinguishing *S. cerevisiae* and *S. bayanus* (20). They are classified from restriction patterns of the *MET2* fragment by digestion with *Pst*I and *Eco*RI. There is a *Pst*I site in the *MET2* sequence of *S. bayanus* and there is none in that of *S. cerevisiae* while the reverse is true for *Eco*RI. Both parental strains and seven fusion products were analyzed by PCR/RFLP of the *MET2* gene (Fig. 3). An *Eco*RI restriction pattern of the two main bands was obtained for EC-1118, but only one band for UCD530 (Fig. 3a). On the other hand, a *Pst*I restriction pattern of the two main bands was obtained for UCD530, but only one band for EC-1118 (Fig. 3a). On an *Eco*RI restriction pattern in EC-1118 and a *Pst*I restriction pattern in UCD530, most slowly migrating bands were caused by incomplete digestion. The restriction pattern of EC-1118 is identical to that of *S. cerevisiae*. UCD530 has the same restriction pattern as that of *S. bayanus*. This finding is in agreement with the observation that UCD530 is defined as *S. bayanus* by AFLP analysis (9). It is expected that a hybrid would have a restriction pattern of three bands upon digestion with both *Eco*RI and *Pst*I (Fig. 3a). All strains had a restriction pattern of three bands except for strain no. 4 (Fig. 3b). These results suggest that they are hybrids between both parental strains except for strain no. 4, although the three bands obtained by *Eco*RI restriction and one band obtained by *Pst*I restriction in strain no. 4 may be caused by mitotic recombination after cell fusion.

The relative DNA content was determined using a flow cytometer (Table 1). The relative DNA content of the fusion products except for strain no. 4 was more than that of the parental strains. The relative DNA content of strain no. 4 was the same as that of the parental strains. These results indicate that in the fusion products, except for no. 4, the chromosomes from parental strains were completely or partially combined. The chromosomal patterns of parental strains and the fusion products obtained were analyzed by CHEF as described in the Materials and Methods. The results are

TABLE 1. Relative DNA content of parental strains and fusion products in wine yeasts

Strain	Relative DNA content	Strain	Relative DNA content
EC-1118	100	No. 4	100
UCD530	100	No. 5	175
No. 1	188	No. 6	156
No. 2	194	No. 7	181
No. 3	200		

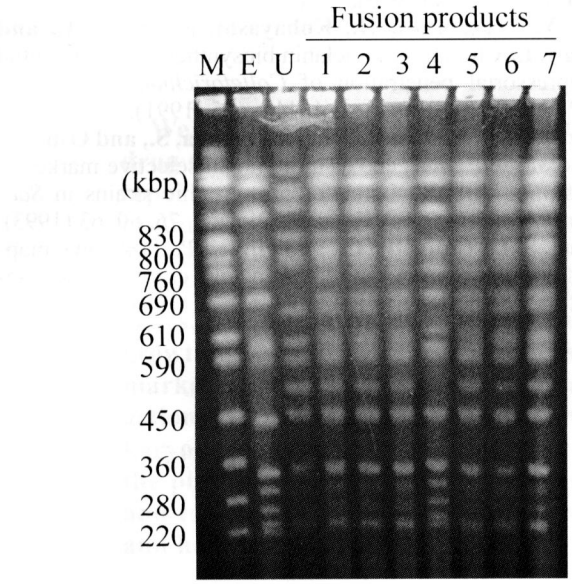

FIG. 4. Electrophoretic karyotypes of parental strains and fusion products. Chromosomal DNAs from EC-1118 (E), UCD530 (U) and fusion products (lanes 1 to 7) were separated by CHEF. Lane M, Chromosomal DNA of YPH149 (23) was run in parallel as size markers.

shown in Fig. 4. The parental strains yielded different patterns (Fig. 4, lanes E and U). While strain no. 4 displayed the same patterns as EC-1118 (Fig. 4, lanes E and 4), in other fusion products a combination of parental bands was observed at 690 kbp (Fig. 4, lanes E to 3 and 5 to 7). Taken together, these results indicated that the fusion products, except for strain no. 4, are hybrids between EC-1118 and UCD530.

At present, Japanese government regulations do not permit the use of strains having recombinant DNA molecules for industrial production. Therefore, the two plasmids used must be cured from hybrids: each of the two hybrid strains was cultivated in liquid YPAD medium at 30°C for 2 d, plated onto YPD plates and incubated at 30°C for 2 to 3 d. Colonies were screened for those which did not grow on YPDM medium containing 0.7 μg/ml of aureobasidin A or 0.3 mg/ml of G418 by the replica plating method. To confirm whether these clones lost their plasmids, DNAs from the clones were prepared and subjected to Southern blot hybridization. The hybridization patterns in the gel showed that strains resistant to both drugs maintained plasmids but sensitive strains did not (Fig. 5). Thus, plasmids used as selection markers are easily eliminated from hybrids. We conclude from all the observations described in this study that this system is useful for the selection of wine yeasts that are homothallic and unable to sporulate.

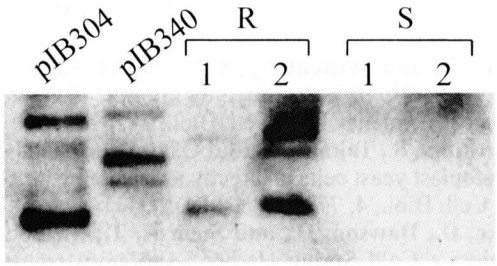

FIG. 5. Southern hybridization analysis for hybrids of wine yeasts resistant or sensitive to drugs. In hybrids no. 1 (lanes R1 and S1) and no. 2 (lanes R2 and S2), DNA was prepared from the cells of resistant (R) and sensitive (S) strains to both G418 and AbA. Plasmids pIB304 and pIB340 were used as controls. A fluorescein-labelled pUC19 digested with *Hin*dIII was used as the probe.

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REFERENCES

1. **Nakazawa, N., Tsuchihara, K., Hattori, T., Akita, K., Harashima, S., and Oshima, Y.:** A method for direct selection of mating-competent clones from mating-incompetent industrial strains of *Saccharomyces cerevisiae*. J. Ferment. Bioeng., **78**, 6–11 (1994).
2. **Nakazawa, N., Okawa, K., Sato, T., Enei, H., and Harashima, S.:** Mass mating method in combination with G418- and aureobasidin A-resistance markers for efficient selection of hybrids from homothallic strains in *Saccharomyces cerevisiae*. J. Biosci. Bioeng., **88**, 468–471 (1999).
3. **Goto, S.:** Wine yeast, p. 44–55. *In* A society for the study of sake yeast (ed.), Study of sake yeast. Brewing Society of Japan, Tokyo (1992).
4. **Mukai, N., Nishimori, C., Wilson Fujishige, I., Mizuno, A., Takahashi, T., and Sato, K.:** Beer brewing using a fusant between a sake yeast and a brewer's yeast. J. Biosci. Bioeng., **5**, 482–486 (2001).
5. **Yamazaki, T. and Nonomura, H.:** Use of chloramphenicol-inherent resistance in protoplast fusion of industrial yeast strains. J. Ferment. Bioeng., **72**, 300–302 (1991).
6. **Yamazaki, T. and Nonomura, H.:** Inherent G418-resistance in hybridization of industrial yeasts. J. Ferment. Bioeng., **77**, 202–204 (1994).
7. **Kucsera, J., Pfeiffer, I., and Ferenczy, L.:** A novel method for hybridization of *Saccharomyces* species without genetic markers. Can. J. Microbiol., **44**, 959–964 (1998).
8. **Kitamoto, K., Oda, K., Gomi, K., and Takahashi, K.:** Construction of uracil and tryptophan auxotrophic mutants from *sake* yeasts by disruption of *URA3* and *TRP1* genes. Agric. Biol. Chem., **54**, 2979–2987 (1990).
9. **Azumi, M. and Goto-Yamamoto, N.:** AFLP analysis of type strains and laboratory and industrial strains of *Saccharomyces sensu stricto* and its application to phenetic clustering. Yeast, **18**, 1145–1154 (2001).
10. **Mukai, Y., Harashima, S., and Oshima, Y.:** AAR1/TUP1 protein, with a structure similar to that of the β subunit of proteins, is required for a1-α2 and α2 repression in cell type con-

trol of *Saccharomyces cerevisiae*. Mol. Cell. Biol., **11**, 3773–3779 (1991).
11. **Stuart, D. and Wittenberg, C.:** Cell cycle-dependent transcription of CLN2 is conferred by multiple distinct cis-acting regulatory elements. Mol. Cell. Biol., **14**, 4788–4801 (1994).
12. **Harashima, S., Takagi, A., and Oshima, Y.:** Transformation of protoplast yeast cells is directly associated with cell fusion. Mol. Cell. Biol., **4**, 771–778 (1984).
13. **Burke, D., Dawson, D., and Stearns, T.:** Methods in yeast genetics: a Cold Spring Harbor Laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2000).
14. **Ito, H., Fukuda, Y., Murata, K., and Kimura, A.:** Transformation of intact yeast cells treated with alkali cations. J. Bacteriol., **153**, 163–168 (1983).
15. **Sambrook, J. and Russell, D. W.:** Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001).
16. **Toh-e, A. and Oshima, Y.:** Characterization of a dominant, constitutive mutation, *PHOO*, for the repressible acid phosphatase synthesis in *Saccharomyces cerevisiae*. J. Bacteriol., **120**, 608–617 (1974).
17. **Schweingruber, M. E., Fluri, R., Maundrell, K., Schweingruber, A.-M., and Dumermuth, E.:** Identification and characterization of thiamin repressible acid phosphatase in yeast. J. Biol. Chem., **261**, 15877–15882 (1986).
18. **Nosaka, K.:** High affinity of acid phosphatase encoded by *PHO3* gene in *Saccharomyces cerevisiae* for thiamin phosphate. Biochem. Biophys., **1037**, 147–154 (1990).
19. **Sheehan, C. and Weiss, S. A.:** Yeast artificial chromosomes: rapid extraction for high resolution analysis. Nucleic Acids Res., **8**, 1043–1059 (1990).
20. **Masneuf, I., Aigle, M., and Dubourdieu, D.:** Development of a polymerase chain reaction/restriction fragment length polymorphism method for *Saccharomyces cerevisiae* and *Saccharomyces bayanus* identification in enology. FEMS Microbiol. Lett., **138**, 239–244 (1996).
21. **Kubo, Y., Nakamura, H., Kobayashi, K., Kino, T., and Foresaw, I.:** Cloning of a melanin biosynthetic gene essential for appressorial penetration of *Colletorichum lagenarium*. Mol. Plant-Microbe Interact., **4**, 440–445 (1991).
22. **Nakazawa, N., Hashimoto, H., Harashima, S., and Oshima, Y.:** Use of the *PDR4* gene as a dominant selective marker in combination with cerulenin for prototrophic strains in *Saccharomyces cerevisiae*. J. Ferment. Bioeng., **76**, 60–63 (1993).
23. **Gerring, S. L., Connelly, C., and Hieter, P.:** Positional mapping of genes by chromosome blotting and chromosome fragmentation. Methods Enzymol., **194**, 57–77 (1991).